

Serum monocyte chemoattractant protein-1 levels and subclinical atherosclerosis in patients with non-alcoholic steatohepatitis

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Abstract. Introduction: The chemokine monocyte chemoattractant protein-1 (MCP-1) is implicated in chronic inflammation, insulin resistance and atherosclerosis. Hepatic inflammation is one of the main pathogenic mechanisms in non-alcoholic steatohepatitis (NASH). Aim: Our study aimed to evaluate serum MCP-1 levels and to investigate the relationship between MCP1, visceral fat thickness (VFT) and cardiovascular risk measured by carotid intima-media thickness (c-IMT) in patients with NASH. Patients and methods: 50 patients with NASH and 30 healthy controls, age and gender matched, were recruited. Lipid profile, liver biochemical markers, serum MCP-1, insulin level, HOMA-IR, VFT and c-IMT were assayed. Results: Patients with NASH had an altered lipid profile and liver biochemical markers; HOMA-IR, VFT, c-IMT and serum MCP-1 levels were significantly higher compared to controls. Also, serum MCP-1 level showed significant positive correlation with waist circumference, body mass index, VFT, HOMA-IR, free cholesterol, triglycerides, LDL-cholesterol, aminotransferases, c-IMT and negative correlation with HDL-cholesterol. Multiple linear regression analysis showed that NASH and HOMA-IR were significantly associated with higher levels of serum MCP-1, this relationship being independent of high c-IMT. Conclusion: Serum MCP-1 levels was statistically significant higher in patients with NASH than in the control group and to this increase contributes both as visceral adipose tissue as hepatic inflammation. In the same time, in patients with NASH the liver may be as well a target and a source of some pro-inflammatory mediators.

Key Words: non-alcoholic steatohepatitis, monocyte chemoattractant protein-1, visceral fat thickness, carotid intima-media thickness, hepatic inflammation.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) represents the most common cause of chronic liver disease in the world and comprises a spectrum of pathologic conditions of different severity, ranging from simple steatosis to steatohepatitis (NASH) which can progress to cirrhosis, in the absence of significant alcohol consumption (Brunt et al 2005; Ratziu et al 2010). NAFLD is associated with increased cardiovascular morbidity and mortality so that cardiovascular disease is the leading cause of death in these patients (Misra et al 2009; Rafiq et al 2009; Söderberg et al 2010).

Although several studies (Kotronen et al 2008; Kim et al 2009) have shown that NAFLD and especially its necroinflammatory subtype – NASH, have a higher cardiovascular risk than that conferred by the presence of the metabolic syndrome alone, the biological mechanisms linking NAFLD and atherosclerosis are still poorly understood.

NAFLD is tightly associated with central obesity and its pathogenesis is currently thought to be a „multiple-hit process”

involving insulin resistance, oxidative stress, lipid peroxidation, release of cytokines and adipokines and inflammation, that plays a central role in NASH (Kim et al 2008, 2009). In the mechanism of liver inflammation, an important role is played by the pro-inflammatory cytokines released both locally and in the visceral adipose tissue that arrive via the portal vein to the liver (Haukeland et al 2006; Clement et al 2008).

The monocyte chemoattractant protein-1 (MCP-1, also known as CCL2) is a small cytokine belonging to the CC-chemokine family that is produced by many cell types but predominantly by macrophages and endothelial cells and is a potent chemotactic factor for monocytes (Deshmane et al 2009). MCP-1 plays a dominant role in the recruitment of inflammatory monocytes and their dedifferentiation into macrophages in adipose tissue (Bruun et al 2005; Dahman et al 2005; Clement et al 2008), liver (Haukeland et al 2006; Kanda et al 2006; Osterreicher et al 2009) and arterial wall (Deshmane et al 2009). Recent studies provided evidence that chemokines are critically involved in acute and chronic liver diseases (Heymann et al 2009; Kalmark et al 2009; Zimmermann et al 2010).

There are very few reported studies that evaluated MCP-1 serum level in NAFLD patients (Haukeland et al 2006; Kanda et al 2006) and the relationship between serum MCP1 levels and sub-clinical atherosclerosis has not been evaluated in patients with biopsy proven NASH. The c-IMT is an established indicator of subclinical atherosclerosis and is used as a surrogate marker for cardiovascular risk (Bonora et al 2003; Kawamoto et al 2005). Thus, the aim of this study was to investigate the relationship between MCP-1, visceral fat thickness (VFT) and cardiovascular risk evaluated by measuring c-IMT in patients with NASH.

Patients and methods

Study participants

Fifty patients with NASH and 30 healthy subjects as control group were enrolled in the study.

The NASH diagnosis was based on previous liver biopsy (NAFLD activity score ≥ 3) (Kleiner et al 2005) and reconfirmed by abdominal ultrasonography, elevated liver enzymes (≥ 1.5 times the upper normal limit) for 6 months before liver biopsy, hepatic steatosis on ultrasound and exclusion of other etiologic factors of chronic liver disease (daily alcohol consumption >30 g for men and >20 g for women, viral and autoimmune hepatitis, hemochromatosis, primary biliary cirrhosis, Wilson's disease, $\alpha 1$ -antitrypsin deficiency, drug-induced liver disease liver carcinoma or liver cirrhosis Other diseases possible associated with increased MCP-1 serum levels were also excluded (i.e. rheumatoid arthritis, pulmonary fibrosis, cancer, multiple sclerosis, kidney diseases). Inclusion criteria for controls were normal liver ultrasound imaging, negative serology for viral hepatitis and normal liver biochemical tests.

The groups were matched in terms of age (NASH patients 45.7 ± 10.9 years vs. control subjects 44.9 ± 7.7 years, $p=0.7$) and gender (70% male patients vs. 66.6 % male control subjects, $p=0.8$).

Before patient recruitment, all participants signed an informed consent the study protocol has been designed in accordance with the WMA Declaration of Helsinki and was approved being approved by the "Iuliu Hațieganu" University of Medicine and Pharmacy Ethics Committee. The informed consent and the research protocol were in accordance with the World Medical Association, Declaration of Helsinki. All participants underwent a complete clinical, laboratory investigation and ultrasound evaluation.

Clinical evaluation

Personal data, demographic characteristics (gender and age), anthropometric (weight, height, waist circumference (WC), waist/hip ratio (WHR)) and clinical data (hepatomegaly, blood pressure) were recorded from each participants. Case history and physical examination provided data on cardiovascular risk factors (e.g. smoking, sedentary lifestyle, history of diabetes, dyslipidemia and high blood pressure). Information on daily alcohol consumption was obtained from all participants by questionnaire. Body weight was measured in light clothing and without shoes to the nearest half kilogram. Standing height was measured in orthostatic, without shoes, using a stadiometer and was expressed in meters (m). Body mass index (BMI, kg/m^2) was calculated by dividing weight in kilograms by the square of height in meters. BMI was determined as normal <25 kg/m^2 ,

overweight corresponded to BMI between 25-30 kg/m^2 , obesity to BMI >30.1 kg/m^2 . Waist circumference was measured in a standing position at the midpoint between the lower border of the rib cage and the iliac crest at the end expiration, whereas hip circumference was similarly obtained at the widest point between hip and buttock. Abnormal W/H ratio was considered if it was > 0.89 (WHO Obesity 2011).

Laboratory investigation

Venous blood sample was drawn in the morning, after an overnight fasting. Plasma liver enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase, total cholesterol, HDL-cholesterol, LDL-cholesterol and fasting glucose were assayed on an automatic analyzer (Konelab 301-Thermo Electron Corp., Finland) using standard laboratory procedures. Serum insulin antibodies were measured by ELISA method, following the kit protocol (Diametra, Italy). The degree of insulin resistance was calculated according to the homeostasis model assessment for insulin resistance (HOMA-IR) using the formula: fasting plasma glucose (mg/dl) x fasting serum insulin ($\mu\text{U}/\text{ml}$)/405 (Matthews et al 1985). The cutoffs of ≥ 2 and >4 were chosen as criteria for insulin resistance and prediabetes, respectively (Romero- Gómez et al 2006).

Serum MCP-1 antibodies were measured using a competitive enzyme-linked immunosorbent assay, following the kit protocol (Ray Bio Human MCP-1 ELISA kit, USA).

Ultrasound evaluation

Each participant underwent abdominal and carotid Doppler ultrasound investigations in order to assess hepatic steatosis, visceral fat thickness (VFT), carotid intima-media thickness (c-IMT) and to ascertain the presence of carotid plaques.

Carotid arteries and hepatic steatosis were evaluated using a high resolution ultrasonographic system Logiq 7 (General Electric, USA) with a 7.5 MHz respectively 5 MHz transducer, by a single trained operator who was blind to clinical characteristics and laboratory findings of participants.

c-IMT measurements were performed in the supine position with the neck extended and the chin turned away from the side being examined according to the Mannheim consensus. Measurements involved a primary transverse and longitudinal scanning of the common carotid artery, bifurcation, and internal carotid. The c-IMT was measured on the far wall at 1 cm from bifurcation of the common carotid artery as the distance between the lumen-intima interface and the media-adventitia interface. The mean c-IMT was calculated by using five values and the maximum c-IMT was considered the highest of these measurements. Values above 0.90 mm were considered increased. The presence of plaques was evaluated in a 30 mm-long segment, both in the left and right common carotids, internal carotid and bulb. The plaque was defined as a focal structure that encroached into the arterial lumen for minimum 0.5 mm or 50% of the surrounding IMT value, or demonstrated a thickness >1.5 mm as measured from media-adventitia interface to the intima-lumen interface (Touboul et al 2007; Ramilli et al 2009).

Hepatic steatosis was defined as the presence of diffuse hyper-echoic texture, bright liver, increased liver echo texture compared to the kidneys, vascular blurring and deep attenuation of

the ultrasonic beam. The VFT was measured with the probe located 1cm above the umbilicus on the xypho-umbilical line in both longitudinal and transverse views and defined as the distance between linea alba and the anterior wall of the aorta (Ramilli *et al* 2009; Sanyal *et al* 2002).

Morphopathology study

All patients diagnosed with NASH had liver biopsies, which were performed under ultrasonographic guidance by the same operator and interpreted by an experienced pathologist blinded to patient history, clinical and laboratory findings. The liver biopsies were graded according to the NAFLD scoring system (NAFLD Activity Score - NAS) proposed by Kleiner *et al* (2005). According to the NAS score, all patients included in this study had a NAS score ≥ 3 .

Statistical analysis

All results were subjected to statistical analyses using SPSS for Windows, version 21.0. (Chicago, Illinois, USA). The continuous data were tested for normality of distribution using Kolmogorov-Smirnov test and were characterized by median and percentiles (25-75%) or by mean \pm standard deviation (SD), when appropriate. Nominal or ordinal variables were characterized using frequencies and percentages. The chi square test (χ^2) was used in order to compare the frequencies of nominal variables. The t test or Mann-Whitney was used to compare continuous variables between the two groups. Spearman's rank correlation was used to assess the relationship between MCP-1 and some cardiovascular risk factors and c-IMT. A multiple linear regression model was used to identify the statistical significance of relationships between selected variables and serum MCP-1 levels. p value < 0.05 was considered statistically significant.

Results

Because of the study design, the age and gender distribution were similar in the NASH and control groups. BMI was positively correlated with age ($r=0.364$; $p=0.001$), WC ($r=0.751$; $p<0.001$) and VFT ($r=0.463$; $p=0.001$).

As expected, serum liver enzymes, insulin levels, HOMA IR, free Cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, VFT were higher in NASH than control group (Table 1). Serum MCP-1 levels were significantly higher in NASH group vs. control group (Table 1) and significantly positively correlated with AST and ALT ($r=0.425$; $p<0.001$, respectively $r=0.302$; $p<0.001$), insulin level ($r=0.324$; $p=0.005$) and HOMA-IR ($r=0.432$; $p<0.001$).

The correlations between some cardiovascular risk factors and serum MCP1 levels are summarized in Table 2.

When considering classical cardiovascular risk factors (i.e. age, gender, smoking, sedentary lifestyle, hypertension, diabetes), only patients with hypertension had significantly higher levels of MCP-1 vs. patients without hypertension [769.2 (453.1, 902.9); 338.3 (287.9, 432.3); $p=0.003$].

To reveal whether serum MCP1 levels are related to markers of lipid profile, correlations were tested between MCP1 and serum levels of four markers. Serum MCP1 levels correlated positively with free cholesterol, LDL-cholesterol, triglycerides and negatively with HDL-cholesterol (Table 2).

Serum MCP-11 was significantly positively correlated with c-IMT ($r=0.432$, $p<0.001$).

Multiple linear regression analysis showed that NASH and HOMA-IR were independently associated with higher levels of serum MCP-1 (β coefficient 0.180, $p=0.003$; respectively β coefficient 0.049, $p=0.002$), this relationship being independent of high c-IMT.

Discussion

The main finding of this study showed that serum MCP-1 levels were statistically higher in NASH patients vs. control group. This observation confirms previous studies investigating the role of CCL2 in NAFLD pathology (Haukeland *et al* 2006; Westerbacka *et al* 2007; Kiviluoto *et al* 2008).

Based on our results, we could presume that two possible mechanisms could be involved in higher serum MCP-1 levels in NASH patients. Firstly, increased serum MCP-1 could be a direct consequence of abdominal obesity and insulin resistance. We found a significant positive association between serum MCP-1 levels and WC, BMI and VFT, suggesting that adipose tissue might be a significant determinant of circulating MCP-1 levels. VFT has been found to be a reliable indicator of the central obesity and it is considered a better predictor of insulin resistance and cardiovascular disease (Freedland *et al* 2004). It is well accepted that the central obesity is associated with low-grade chronic inflammation characterized by macrophage infiltration of adipose tissue and increased release of pro-inflammatory cytokines (TNF- α , IL-6, MCP-1, etc), which play a crucial role in the development of insulin resistance, metabolic syndrome and atherosclerosis (Weisberg *et al* 2003; Despres *et al* 2008). The most important factor involved in the chemotactic recruitment of circulating monocytes to the adipose tissue is MCP-1/CCL2 (Weisberg *et al* 2003; Kanda *et al* 2007).

The expression of MCP-1 is higher in visceral fat, as compared with subcutaneous adipose tissue (Bruun *et al* 2005).

The pathogenesis of NASH is closely related to the presence of insulin resistance. In the present study HOMA-IR was an independent predictor of increased MCP-1 levels in patients with NASH. This association has been shown in mice, baboons and humans (Kamei *et al* 2006; Kim *et al* 2006; Bose *et al* 2009; Voruganti *et al* 2009).

Our results suggested that insulin resistance may be both a cause and an effect of increased MCP-1. Insulin resistance-induced obesity is associated with increased lipolysis in the adipose tissue and resulted free fatty acids (FFA) represent the trigger for macrophage activation, followed by the release of pro-inflammatory cytokines (including MCP-1) in both VAT and the liver. Also there are studies showing that MCP-1 contributes to the appearance of insulin resistance induced obesity. Thus, absence of MCP-1 receptor in mice fed with high-fat diet decreases insulin resistance and hepatic steatosis (Weisberg *et al* 2006). On the other hand, mice over expressing CCL2 in adipose tissue presented increased insulin resistance and TG levels (Kanda *et al* 2006).

Increased FFA and pro-inflammatory molecules in the portal vein may cause impaired liver function (Girard *et al* 2008).

Several studies have emphasized the crucial role of infiltrating monocytes/macrophages for the progression of liver inflammation and fibrosis in experimental mouse models (Kalmarm *et al*

Table 1. Baseline characteristics of the study participants

| Variables | NASH group (n=50) | Control group (n=30) | p |
|---------------------------------|----------------------|-------------------------|---------|
| Age (years) | 45.76±10.9 | 44.9±7.79 | 0,7 |
| Gender (Male/Female) | 35/15 | 20/10 | 0,8 |
| High blood pressure | 25 (51%) | 0 | <0.001 |
| Diabetes mellitus (number; %) | 5 (10.2%) | 0 | 0,1 |
| Sedentary lifestyle (number; %) | 27 (55.1%) | 7 (23.1%) | 0,01 |
| Smokers (%) | 15 (30.6%) | 3 (10%) | 0,06 |
| BMI (kg/m ²) | 31.09±4.6 | 22.8±3.6 | <0.001 |
| WC (cm) | 106.1±12.6 | 80.87±12.1 | <0.001 |
| WHR | 1.02 ± 0.09 | 0.85 ± 0.08 | <0.001 |
| Free cholesterol (mg/dl) | 220.18±47 | 178.6±20,1 | <0.001 |
| HDL-cholesterol (mg/dl) | 52.14±15.3 | 65.73±14.9 | <0.001 |
| LDL-cholesterol (mg/dl) | 142.27±38.8 | 110.93±23.5 | <0.001 |
| Triglycerides (mg/dl) | 130 (98; 267) | 77 (56; 110) | 0,002 |
| Fasting plasma glucose (mg/dl) | 95 (88.2;106) | 89.5 (86; 95) | 0,05 |
| Insulin (µU/ml) | 11.86 (8.5; 18,9) | 7.89 (5.9; 10.7) | 0,004 |
| HOMA-IR | 2.81 (1.88; 4.87) | 1.71 (1.35; 2.35) | 0,001 |
| HOMA-IR >2 | 30 (70%) | 13 (43.3%) | 0,04 |
| AST (IU/L) | 38 (31; 56) | 21 (17; 23) | <0.001 |
| ALT (IU/L) | 55 (41; 78) | 20 (17; 22) | <0.001 |
| γ -GT (IU/L) | 59 (38; 77) | 23 (18; 28) | <0.001 |
| MCP-1(pg/ml) | 567.1 (317.1; 889.1) | 306.2 (276.4; 429.3) | <0.001 |
| VFT(mm) | 87.51±21.1 | 50±16.1 | <0.001 |
| c-IMT (mm) | 0.9 (0.7; 0.9) | 0.6 (0.7; 0.8) | < 0.001 |
| Elevated c-IMT (number; %) | 42 (84%) | 6 (20%) | <0.001 |
| Carotid plaques (number; %) | 7 (14%) | 0 | 0,04 |

2009; Duffield *et al* 2005) and in patients with liver cirrhosis (Zimmermann *et al* 2010).

In conditions of liver damage, C-C motif chemokine receptor (CCR)-2 and its ligand, MCP-1/CCL-2, promote monocyte subset infiltration into the liver (Kalmark *et al* 2009; Seki *et al* 2009). The association between MCP-1 and NAFLD is reliable with the data published by Kanda *et al* (2007) which showed that insulin resistance and hepatic steatosis were improved as a result of the MCP-1 gene deletion in mice that were fed a high fat diet. In this study, NASH patients had both a c-IMT and serum MCP-1 levels significantly higher than control group and c-IMT was positively associated with MCP-1. In multiple regression analysis, NASH and HOMA-IR were independently associated with higher levels of serum MCP-1, this relationship being independent of c-IMT. Although it is known that MCP-1 plays a crucial role in initiating atherosclerosis by recruiting monocytes into the subendothelial cell layer (Ross *et al* 1999) and expression of the MCP-1 mRNA is markedly increased in atherosclerotic lesions (Yla-Herttuala *et al* 1991) our results showed that

increased serum MCP-1 levels are not a direct consequence of atherosclerosis development.

We observed that increased serum MCP-1 was associated with a pro-atherogenic lipid profile characterized by low HDL-cholesterol, high LDL-cholesterol, free cholesterol and triglycerides in NASH patients. This observation corresponds with another study reporting that MCP-1 levels are increased in patients with hypercholesterolemia (Han *et al* 1999) and hypertriglyceridemia (Ibrahim *et al* 2012).

In animal studies, it has been reported that hepatic inflammation and insulin resistance may contribute to the development of dyslipidemia and increased susceptibility to atherosclerosis (Biddinger *et al* 2008; Arbones-Mainaret *et al* 2008).

Regarding classical cardiovascular risk factors, serum MCP-1 levels were significantly correlated with hypertension, obesity and dyslipidemia, but not with age, gender, diabetes, sedentary lifestyle or smoking.

Although our study included only few diabetic patients, our results are consistent with those that were conducted in a large population-based survey that showed that serum MCP-1 levels

Table 2. Spearman's correlations between serum MCP-1 levels and some cardiovascular risk factors

| | r | P |
|--------------------------------|--------|--------|
| Age (years) | 0.174 | 0.1 |
| BMI (kg/m ²) | 0.405 | <0.001 |
| WC (cm) | 0.371 | 0.001 |
| WHR | 0.341 | 0.003 |
| VFT | 0.396 | <0.001 |
| Free cholesterol (mg/dl) | 0.332 | <0.001 |
| HDL-cholesterol (mg/dl) | -0.399 | <0.001 |
| LDL-cholesterol (mg/dl) | 0.254 | 0.02 |
| Triglycerides (mg/dl) | 0.412 | <0.001 |
| Fasting plasma glucose (mg/dL) | 0.09 | 0.4 |
| Insulin (μU/mL) | 0.425 | <0.001 |
| HOMA IR | 0.432 | <0.001 |
| Smoking | 0.104 | 0.3 |
| Sedentary lifestyle | 0.13 | 0.2 |

were not associated with impaired glucose tolerance or type 2 diabetes (Herder et al 2006).

There are several limitations of this study. First, it is a cross-sectional study, with all the deriving consequences. Second, the number of subjects is relatively small to draw definite conclusions. To our knowledge, this is the first study of patients with NASH aiming to establish complex relationship between serum MCP-1 level, insulin resistance, VFT and early carotid lesions measured by c-IMT.

Conclusion

The present study showed that serum MCP-1 levels were statistically significant higher in patients with NASH than in the control group, to this increase contributing both VFT and liver inflammation. At the same time, in NASH patients the liver is both a target and a source of pro-inflammatory factors.

The development of MCP-1 receptor blocking agents would represent a novel therapeutical strategy for NASH patients.

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